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(54) Title: FUNCTIONAL INTERACTIONS BETWEEN GLIAL S-100$_B$ AND CENTRAL NERVOUS SYSTEM SEROTONERGIC NEURONS

(57) Abstract

The production of S-100$_B$ in a subject is stimulated by administering an effective amount of an agonist acting on the 5HT$_{1a}$ receptor, the growth of central serotonergic neurons is stimulated by contacting the neurons with an effective amount of S-100$_B$, and the growth of central serotonergic neurons is inhibited by contacting the neurons with an effective amount of an inhibitor of S-100$_B$ production or action. Furthermore, diseases associated with decreased central serotonergic innervation or activity including autism, depression, anxiety, biological rhythm-based sleep disorder, and cortical brain damage may be treated by such methods. Diseases associated with increased central serotonergic innervation but ineffective astroglial S-100$_B$ release, such as Down's Syndrome and Alzheimer's Disease may be treated by up-regulating astroglial 5HT$_{1a}$ receptors, followed by stimulation of S-100$_B$ production or release using a 5HT$_{1a}$ receptor agonist.

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FUNCTIONAL INTERACTIONS BETWEEN GLIAL S-100$_B$ AND CENTRAL NERVOUS SYSTEM SEROTONERGIC NEURONS

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BACKGROUND OF THE INVENTION

Field of the Invention

The invention in the field of neuroscience and medicine relates to a cortical growth factor produced by astroglial cells which is trophic for cortical and serotonergic neurons in the brain.

Description of the Background Art

Serotonergic neurons, which release serotonin (5-hydroxytryptamine, 5-HT) as a neurotransmitter, play a key role in the general maturation of the brain. Changes in the innervation density of serotonergic nerve fibers would be expected to induce changes in the maturation of "target" neurons with which the serotonergic fibers communicate.

Evidence exists for a brain serotonergic growth factor (SGF). For example, destruction of serotonergic nerves by intracerebral injection of 5,7-dihydroxytryptamine (5,7-DHT) promotes the homotypic collateral sprouting of hippocampal serotonergic fibers (Azmitia, E.C., et al., Nature 274:374-377 (1978); Zhou et al., Brain Res. 373:337-348 (1986)), and enhances outgrowth of transplanted fetal serotonergic neurons (Zhou et al., J. Neurosci. Res., 17:235-248 (1987)). A high speed supernatant prepared from 5,7-DHT-lesioned hippocampus could stimulate fetal serotonergic neurons both in culture (Azmitia et al., Soc. Neurosci. Abstr. 12 (1986)) and upon transplantation into the adult cerebellum (Zhou et al., Brain Res. 507:301-308 (1990)), suggesting that the trophic substance is a soluble protein.

Binding studies with appropriately labelled ligands initially revealed the existence of two major types of serotonin receptors in the brain, termed 5-HT_1 and 5-HT_2, and later pointed to the existence of further subtypes, such as 5-HT_{1a} and 5-HT_{1b} (Peroutka, S.J. et al., Feder. Proc. 42:212-217 (1983); Pazos, A. et al., Brain Res. 205:346 (1985)). Other types of 5-HT receptors (e.g., 5-HT_3 and 5-HT_4) have been described more recently (see: Whitaker-Azmitia, P.M. et al., Eds., Ann. N.Y. Acad. Sci. volume 600 (1990)).

Serotonin neurons have been shown to regulate their own development, i.e. to "autoregulate" (Whitaker-Azmitia, P.M. et al., Neurosci. Lett. 67:307-312 (1986)), due in part to release of growth factors by stimulation of 5-HT_{1a} receptors on astrocytes (Whitaker-Azmitia, P.M. et al., J. Neurochem. 46:1186-91 (1986)). Glial cells, such as astrocytes, have 5-HT receptors (Whitaker et al., ibid.). The conditioned medium from glial cells which had been exposed to a selective agonist for the 5-HT_{1a} receptor subtype stimulated cultured serotonergic neurons (Whitaker-Azmitia et al., Brain Res. 497:80-85 (1989)). These results suggest the existence of a soluble hippocampal SGF of glial origin.

A number of proteins have been implicated as neuronal growth factors. Nerve growth factor (NGF) appears to act as a CNS cholinergic growth factor (Hefti, J. Neurosci. 6:2155-2162 (1986)). Epidermal growth factor (EGF) has trophic effects on neuron-like PC-12 cells (Leonard et al., Mol. Cell. Biol. 7:3156-3167 (1987); Isobe, T. et al., J. Neurochem. 43:1494-1496 (1984)). Insulin has been shown to mediate growth of cultured fetal neurons (Heindenreich, K.A. et al., Endocrinology 125:1451-1457 (1989)). The protein S-100_B, which is composed of two ß-subunits, stimulates neurite extension in cultured chick cortical neurons, hence its designation as a "cortical" growth factor (Kligman D., et al., Proc. Natl. Acad. Sci. U.S.A. 82:7136-7139 (1985)). Calmodulin has been shown to

have substantial structural homology and a similar Ca^{2+} binding profile to S-100_B (Isobe, T., et al., Endocrinology 125:1451-1457 (1989))

A family of proteins named S-100 was first isolated nearly 25 years ago (Moore, B.W., Biochem. Biophys. Res. Commun. 19:739-742 (1965)). The member of this family designated S-100_B was known to promote neurite extension in chick embryo cultures (Kligman, D. et al., Proc. Natl. Acad. Sci. USA 82:7136-39 (1985)). S-100 production was stimulated in cultures of the rat astroglioma line, C6, by dibutyryl cyclic AMP (Labourdette, G. et al., Biochem. Biophys. Res. Comm. 96:1702-09 (1965)). Furthermore, S-100 may be releasable from brain tissue (Shashoua, V.E. et al., J. Neurochem. 42:1536-41 (1984)).

The well-known factor NGF has no effect of 5-HT sprouting in damaged hippocampal neurons (Kiedrowsk L., et al., Eur. J. Neurochem. 43:1494-1496 (1984)), or in cultured dopaminergic neurons (Dreyfus, C.F., et al., Brain Res. 194: 540-547 (1980)).

During fetal brain development, S-100_B, as detected by specific antibodies, shows an intense yet transient rise in the midline raphe region, where the serotonergic neurons are developing (Van Hartesveldt, C.J. et al., J. Comp. Neurol. 253:175-184 (1986)).

Finally, the human gene for the ß subunit of S-100 has been mapped to the distal half of the long arm of chromosome 21, a candidate region associated with the pathology of Down's Syndrome and Alzheimer's Disease (Allore, R. et al., Science 239:1311-1313 (1988)). This gene was recently shown to contain the cAMP responsive element, CRE, on the promoter region (Allore, R. et al., J. Biol. Chem. 265:15537-15543 (1990)). Furthermore, recent studies have shown an increase in S-100 immunoreactivity in postmortem Alzheimer's Disease and Down's Syndrome brains (Griffin, W.S.T. et al., Proc. Natl. Acad. Sci. USA 86:7611-7615 (1989)). Therefore, greater understanding of the physiological actions of S-100 and knowledge of which

factors regulate its release are believed to be important for the understanding and treatment of both disorders.

SUMMARY OF THE INVENTION

As stated above, the present inventors conceived and conclusively demonstrated that S-100$_B$ is the protein released by astroglial cells upon stimulation of their 5-HT$_{1a}$ receptors which promotes the growth of serotonergic and cortical neurons. Therefore, they have developed methods for manipulating S100$_B$, and through it, serotonergic and cortical neuronal growth and maintenance.

The present invention is directed to a method for stimulating the production of S-100$_B$ in a subject, comprising administering an effective amount of an agonist acting on the 5-HT$_{1a}$ receptor. The 5HT$_{1a}$ agonists useful in the present invention include 5-hydroxytryptamine, 5-methoxytryptamine, buspirone, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), ipsaspirone, gepirone, SM23997, lysergic acid diethylamide, and agonistic antibodies.

The present invention is further directed to a method for stimulating growth of central serotonergic neurons in a subject comprising administering to the subject an effective amount of S-100$_B$, a functional derivative thereof, or an agonist acting at the 5-HT$_{1a}$ receptor, such as those described above.

The present invention includes a method for stimulating the growth of central serotonergic neurons in vitro or in vivo by contacting the neurons with S100$_B$ or a functional derivative thereof. For treatment of diseases associated with dysregulation of serotonergic neurons, in vivo contacting is preferred.

Alternatively, the present invention involves a method for inhibiting the growth of central serotonergic neurons comprising contacting the neurons with an effective amount of an inhibitor of S-100$_B$ production or action. The

inhibitor may be an antibody specific for S-100$_B$ or a 5-HT$_{1a}$ receptor antagonist, such as spiperone and spiroxatine.

The present invention relates to a method for treating a disease associated with decreased central serotonergic innervation or activity in a subject comprising administering an effective amount of S-100$_B$, a functional derivative thereof or a 5-HT$_{1a}$ agonist. Diseases for which this method is useful include autism, depression, anxiety, biological rhythm-based sleep disorder, and cortical brain damage, as well as the treatment of neuronal loss associated with normal aging.

The present invention is directed to a method for treating a disease associated with increased central serotonergic innervation or activity in a subject comprising administering an effective amount of an inhibitor of S-100$_B$ production or action. Such inhibitors include an antibody specific for S-100$_B$ and a 5-HT$_{1a}$ receptor antagonist.

In another embodiment, the invention is directed to a method for stimulating cortical neuronal growth in a subject having Alzheimer's disease comprising the steps of:

(a) up-regulating the expression of 5HT$_{1a}$ receptors on astroglial cells in the brain of the subject; and

(b) stimulating the release of S-100$_B$ in the subject according to the methods described above,

thereby stimulating the cortical neuronal growth.

Diseases for which this method is useful include Down's Syndrome and Alzheimer's Disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a histogram showing the effects of EGF (0.5μg/ml), NGF (10 μg/ml), S-100$_B$ (10 μg/ml) and insulin (100 μg/ml) added daily in 5-fold serial dilutions (most concentrated dose at right) on serotonin uptake. Each bar is the mean ± S.E.M. (n=4) of [^{3}H]5-HT uptake as a percent of

control. Control uptake was 5038 ± 606 cpm for S-100$_B$/insulin and 6159 ± 318 cpm for NGF/EGF.

Figure 2 is a graph showing the effects of a single application of S-100$_B$ and calmodulin on [^{3}H]5-HT uptake capacity after 3 days. Final concentration is shown on the abscissa. Each point represents the mean ± S.E.M. (n=4).

Figure 3 is a histogram showing a morphometric analysis of the total neurite length for individual 5-HT-immunoreactive neurons after 30 h of stimulation. The bars represent the mean ± S.E.M. for 10 neurons in each well (number shown under bars).

Figure 4 is a graph showing effects of native bovine S-100 and of media from astroglial cells stimulated with the selective 5-HT$_{1a}$ agonist ipsapirone (GCM-IPS) on the growth of serotonergic neurons in culture as determined by selective uptake of ^{3}H-serotonin. Hatched bars indicate the effects of S-100 and GCM-IPS in the presence of an antibody to S-100 (final dilution 1/10,000). Each bar represents the mean ± S.E.M. of 4 cultures, derived from different litters. S-100, GCM-IPS and the antibody were all added at time of plating and the growth assessed 3 days later.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The inventors have discovered that S-100$_B$ is a serotonergic growth factor (SGF). Since this protein has no effect on cholinergic and noradrenergic neurons, nor on cells in the peripheral nervous system, it can be said to be specific for central serotonergic nerves.

Serotonin neurons have been shown to autoregulate their own development (Whitaker-Azmitia, P.M. et al., Neurosci. Lett. 67:307-312 (1986)). The present inventors first discovered that this autoregulatory circuit involves the release of a growth factor or factors induced by stimulation of 5-HT$_{1a}$ receptors on astrocytes (Whitaker-Azmitia, P.M. et al., J. Neurochem. 46:1186-91 (1986)).

The present invention is based on the discovery by the present inventors that the growth factor released in response to 5-HT_{1a} receptor stimulation is S-100_B. Therefore, the present invention is directed to the use of 5-HT_{1a} agonists or antagonists as therapeutics, acting via the regulation of S-100_B production and/or release from astroglial cells.

The present invention is directed to methods involving the use of the S-100_B protein, which is a dimer of two ß chains, and is found exclusively in the brain, in contrast to α-α or α-ß dimers, which are also found outside the brain. Also included within the scope of the present invention are functional derivatives of the S-100_B protein.

By "functional derivative" is meant a "fragment," "variant," "analog," or "chemical derivative" of S-100_B, which terms are defined below. A functional derivative retains at least a portion of the function of the S-100_B which permits its utility in accordance with the present invention, namely serotonergic or cortical growth factor activity.

A "fragment" of the S-100_B refers to any subset of the molecule, or of the ß chain, such as a shorter peptide.

A "variant" of the S-100_B refers to a molecule substantially similar to either the entire peptide or a fragment thereof. Variant peptides may be conveniently prepared by direct chemical synthesis of the variant peptide, using methods well-known in the art or by recombinant DNA technology. Amino acid sequence variants of the S-100_B molecule can also be prepared by mutations in the DNA. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence. Any combination of deletion, insertion, and substitution may also be made to arrive at the final construct, provided that the final construct possesses the desired activity, without placing the sequence out of reading frame and preferably not creating complementary

regions that could produce secondary mRNA structure (see EP Patent Application Publication No. 75,444). At the genetic level, these variants ordinarily are prepared by site-directed mutagenesis of nucleotides in the DNA encoding the S-100$_B$ molecule, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. The variants typically exhibit the same qualitative biological activity as the naturally occurring analog.

An "analog" of S-100$_B$ refers to a non-natural molecule substantially similar to either the entire molecule or a fragment thereof.

A "chemical derivative" of S-100$_B$ contains additional chemical moieties not normally a part of the protein or peptide. Covalent modifications of the protein are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues.

Also included in the scope of the invention are salts of the proteins and peptides of the invention. As used herein, the term "salts" refers to both salts of carboxyl groups and to acid addition salts of amino groups of the protein or peptide molecule. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases such as those formed for example, with amines, such as triethanolamine, arginine, or lysine, piperidine, procaine, and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid.

By the term "agonist" is intended any chemical or biological substance capable of binding to a particular receptor, such as the 5-HT$_{1a}$ receptor according to the

present invention, and stimulating a biological response associated with the receptor. The term is intended to include an endogenous molecule which exerts its physiological action by receptor binding and triggering of a signal to a cell, as well as an exogenous agent which mimics the action of such an endogenous agonist. Thus, if a receptor is linked to a second messenger system that signals a positive response, such as, for example, the increased production and/or secretion of a protein growth factor, an agonist will induce production and/or secretion of the growth factor. In contrast, if a receptor is linked to a second messenger system which signals a negative response, such as a termination of cell growth, an agonist for that receptor will inhibit cell growth.

Agonists for 5-HT_{1a} receptors are known in the art and include, but are not limited to, 5-hydroxytryptamine (serotonin), 5-methoxytryptamine, buspirone (U.S. Patent 3,717,634), 8-hydroxydipropylamineotetralin, ipsaspirone (EPO Publication 129,128A), gepirone (U.S. Patent 4,423,049), SM23997 (U.S. Patent 4,507,303), MDL 72832 (U.S. Patent 4,612,312) and lysergic acid diethylamide. In addition, newer polycyclic aryl- and heteroarylpiperazinyl imides with 5-HT_{1a}-binding and activating properties, such as WY-47,846 (cpd. 34) and other disclosed in Abou-Gharbia, M. et al., J. Med. Chem. 31:1382-1392 (1988), may be useful in the present invention. (All of the above references to agonists are hereby incorporated by reference).

In addition, an antibody to the 5-HT_{1a} receptor which by virtue of its epitope specificity stimulates a response, rather than inhibiting the binding of an agonist, termed an "agonistic antibody," is also an agonist as intended herein. The present invention is intended to encompass additional 5-HT_{1a} receptor agonists, including those not yet discovered.

By the term "antagonist" is intended a substance which is itself devoid of intrinsic pharmacologic activity and stimulates no biological response when bound to a

receptor, but has the capacity to bind to a receptor and thereby inhibit binding of, or action of, an agonist. Typically, antagonists act by competing for agonist binding to a receptor.

Antagonists for 5-HT_{1a} receptors are known in the art and include, but are not limited to, spiperone and spiroxatine. In addition, an antibody specific for the 5-HT_{1a} receptor which does not have agonist activity, but inhibits binding or action of an agonist, is also an antagonist, as intended herein. In addition, newer polycyclic aryl- and heteroarylpiperazinyl imides with 5-HT_{1a}-binding properties which would inhibit binding of endogenous agonists (Abou-Gharbia, M. et al., supra) may be useful as antagonists in the present invention.

The principles of receptors, agonists, and antagonists are described, for example, in Gilman, A.G. et al., Goodman and Gilman's "The Pharmacological Basis of Therapeutics", Seventh Edition, Macmillan Publishing Co., New York, 1985, which is hereby incorporated by reference.

The antibodies of the present invention are those which are specific for, and interact with, S-100_B or with 5TH1a receptors and modulate the action of the S-100_B-serotonergic neuron autoregulatory system.

The term "antibody" is meant to include polyclonal antibodies, monoclonal antibodies (mAbs), chimeric antibodies, and anti-idiotypic (anti-Id) antibodies. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen.

Monoclonal antibodies are a substantially homogeneous population of antibodies to specific antigens. MAbs may be obtained by methods known to those skilled in the art. See, for example Kohler and Milstein, Nature 256:495-497 (1975) and U.S. Patent No. 4,376,110. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, GILD and any subclass thereof. The hybridoma producing the mAbs of this invention may be

cultivated in vitro or in vivo. Production of high titers of mAbs in vivo production makes this the presently preferred method of production. Briefly, cells from the individual hybridomas are injected intraperitoneally into pristane-primed BALB/c mice to produce ascites fluid containing high concentrations of the desired mAbs. MAbs of isotype IgM or IgG may be purified from such ascites fluids, or from culture supernatants, using column chromatography methods well known to those of skill in the art.

Chimeric antibodies are molecules different portions of which are derived from different animal species , such as those having variable region derived from a murine mAb and a human immunoglobulin constant region. Chimeric antibodies and methods for their production are known in the art (Cabilly et al, Proc. Natl. Acad. Sci. USA 81:3273-3277 (1984); Morrison et al., Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984); Boulianne et al., Nature 312:643-646 (1984); Neuberger et al., Nature 314:268-270 (1985); Taniguchi et al., European Patent Application 171496 Robinson et al., International Patent Publication #PCT/US86/02269; Liu et al., Proc. Natl. Acad. Sci. USA 84:3439-3443 (1987); Better et al., Science 240:1041-1043 (1988)). These references are hereby incorporated by reference.

An anti-idiotypic (anti-Id) antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody. An Id antibody can be prepared by immunizing an animal of the same species and genetic type (e.g. mouse strain) as the source of the mAb with the mAb to which an anti-Id is being prepared. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants (the anti-Id antibody).

The anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The

anti-anti-Id may be epitopically identical to the original mAb which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against S-100_B, for example, may be used to induce anti-Id antibodies in suitable animals, such as BALB/c mice. Spleen cells from such immunized mice are used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional BALB/c mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for a S-100_B epitope.

The anti-Id mAbs thus have their own idiotypic epitopes, or "idiotopes" structurally similar to the epitope being evaluated, such as an S-100_B epitope, and possessing biological activity of S-100_B.

The term "antibody" is also meant to include both intact molecules as well as fragments thereof, such as, for example, Fab and F(ab')$_2$, which are capable of binding antigen. Fab and F(ab')$_2$ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl et al., J. Nucl. Med. 24:316-325 (1983)).

It will be appreciated that Fab and F(ab')$_2$ and other fragments of the antibodies useful in the present invention may be used according to the methods disclosed herein for intact antibody molecules. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')$_2$ fragments).

An antibody is said to be "capable of binding" a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. The term "epitope" is meant to refer to that portion of any

molecule capable of being bound by an antibody which can also be recognized by that antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics.

An "antigen" is a molecule or a portion of a molecule capable of being bound by an antibody which is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen may have one, or more than one epitope. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

The preferred animal subject of the present invention is a mammal. By the term "mammal" is meant an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects.

By the term "treating" is intended the administering to subjects of S-100$_B$, a functional derivative thereof, or an agonist or antagonist of the 5-HT$_{1a}$ receptor, for purposes which may include prevention, amelioration, or cure of the diseases discussed below.

For example, administration may be by parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Preferred routes for administration of substances which do not cross the blood-brain barrier (such as proteins and larger peptides) to subjects with fully developed blood-brain barriers include intracranial and intracerebroventricular (i.c.v.) routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

According to the present invention, an antibody to S-100$_B$ or to a 5-HT$_{1A}$ receptor is administered prenatally, neonatally or to an adult. For prenatal administration, the antibody is given systemically to the pregnant female or is introduced in utero, for example, into the amniotic cavity. For neonatal treatment, an antibody is administered systemically, for example, by intravenous or intraperitoneal injection. The antibody can cross the blood-brain barrier and enter the brain from the circulation in a young individual in whom the blood-brain barrier is not completely formed, as is well-known to those of skill in the art. In an individual with a fully formed blood-brain barrier, as in an adult, in order to be effective, the antibody, according to the present invention, must be administered intracranially (i.c.), preferably into the cerebral ventricles (i.c.v.) via a cannula, using methods well-known in the art.

Similarly, the S-100$_B$ protein or a functional derivative thereof, is administered prenatally, neonatally, or to an adult as described above for an antibody or fragment thereof. In addition to the pharmacologically active compounds, such as the 5-HT$_{1A}$ agonists and antagonists, S-100$_B$ and functional derivative thereof, or antibodies, the present invention contemplates pharmaceutical preparations which may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Preferably, the preparations, particularly those preparations which can be administered orally and which can be used for the preferred type of administration, such as tablets, dragees, and capsules, and also preparations which can be administered rectally, such as suppositories, as well as suitable solutions for administration by injection or orally, contain from about 0.01 to 99 percent, preferably from about 20 to 75 percent of active compound(s), together with the excipient.

Suitable excipients are, in particular, fillers such as saccharides, for example, lactose or sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example, tricalcium phosphate or calcium hydrogen phosphate, as well as binders such as starch paste, using, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone. If desired, disintegrating agents may be added such as the above-mentioned starches and also carboxymethyl-starch, cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate. Auxiliaries are, above all, flow-regulating agents and lubricants, for example, silica, talc, stearic acid or salts thereof, such as magnesium stearate or calcium stearate, and/or polyethylene glycol.

A number of major developmental disorders involve central serotonergic systems. Autism and Down's Syndrome (DS) are associated with altered serotonergic forebrain innervation, as seen upon postmortem examination of brains from patients with this disorder (Anderson, G.M. et al., Ann. N.Y. Acad. Sci. 600:331-342 (1990)). Hyperserotoninemia has been observed in autistic children and an antibody specific for the human cortical 5-HT_{1a} receptor has been identified in an autistic patient (Anderson, G.M. et al., supra). The present invention provides a means for treating an individual affected by autism or DS in early developmental stages, for example, while in utero. Thus, for example, S-100_{β}, a functional derivative thereof, or 5-HT_{1a} agonist is administered to a pregnant woman carrying a fetus diagnosed as having autism or DS.

Alzheimer's Disease (AD) shares a number of pathophysiological similarities to DS and is also associated with Chromosome 21 (Allore et al., 1988, supra). Brains of AD patients have increased levels of S-100_{β}, in particular in astrocytes (Griffin, W.S.T. et al., supra), which is not

normally releasable. AD patients also have increased serotonergic innervation. As a result of the increased presence of 5HT, astroglial $5HT_{1A}$ receptors are down-regulated.

The present invention provides a method for treating AD by manipulating the serotonergic system to cause release of $S\text{-}100_B$ to stimulate growth of cortical neurons. The method is directed to first up-regulating the astroglial $5HT_{1A}$ receptors in order to render them sensitive to 5HT or exogenous agonists, and then to treating with the agonists in order to stimulate $S\text{-}100_B$ release. Up-regulation of the $5HT_{1A}$ receptors is accomplished by any of a number of means known in the art for depleting central 5HT or blocking its action. Such means include depleting stored serotonin from nerve terminals by agents such as reserpine, fenfluramine or methylene deoxymethamenpetamine (MDMA) (Whitaker-Azmitia, P.C. et al., Eds, Ann. N.Y. Acad. Sci., Vol. 600 (1990)), dietary changes which lower central serotonin levels, such as a tryptophan-deficient diet, or drugs which inhibit 5HT biosynthesis such as parachlorophenylalanine.
Alternatively, since central 5HT release is dependent on intact brain corticosteroids, any treatment which blocks brain corticosteroid levels (for example, synthesis inhibitors such as metapyrone or aminoglutethamide) or corticosteroid action (such as corticosteroid antagonists which are well-known in the art) would also be useful in up-regulating $5HT_{1A}$ receptors. Treatment with $5HT_{1A}$ receptor antagonists, such as those described above, may also achieve the same effect. Such treatment must be performed for a period of time ranging from about 3 days to about 4 weeks. Depending on the drug or means chosen to up-regulate the receptors, and the age, weight and health of the subject, one of ordinary skill in the art will be able to determine the appropriate dose and time course of treatment.

Once the $5HT_{1A}$ receptors have been appropriately up-regulated, resulting in regained sensitivity to agonist action, the treatment method of the present invention

involves providing to the brain a $5HT_{1A}$ receptor agonist such as those described above, preferably by the oral route, in a dose range of about 1 μg/kg to about 10 mg/kg, for a duration of about 3 days to about 4 weeks, in order to release the $S\text{-}100_B$ accumulating in the astroglia. The released $S\text{-}100_B$ then acts as a cortical growth factor to stimulate growth of cortical neurons deficient in AD.

In fact, any pathological process associated with loss of cortical neurons, or serotonergic neurons or their activity, or lack of normal maintenance of serotonergic innervation, can be treated according to the methods of the present invention. Such neuronal loss may accompany normal aging. The present invention is thus directed to a method of treating neuronal loss in an aging individual with $S\text{-}100_B$, a functional derivative thereof, or a $5\text{-}HT_{1A}$ agonist which stimulates $S\text{-}100_B$ production by astroglial cells in situ. If necessary, as described for AD, prior treatment may be used to up-regulated $5HT_{1A}$ receptors in order to allow stimulation of $S\text{-}100_B$ release.

Affective disorders, in particular depression, are diseases with an important serotonergic component. In fact, the mode of action of many effective antidepressant drugs is considered to occur via inhibition of 5HT re-uptake, thus prolonging the availability of 5HT to act on post-synaptic 5HT receptors. Whereas uptake blockade can occur within minutes of treatment, therapeutic benefits are typically seen only after prolonged (e.g. 3 weeks) of antidepressant therapy (see, for example, Gilman et al., supra). This difference in time courses suggested to the present inventors that depression involves alterations in levels of serotonergic innervation and synapse formation. Thus, according to the present invention, stimulation of serotonergic neuronal growth by treatment with $S\text{-}100_B$, a functional derivative thereof, or a $5\text{-}HT_{1A}$ agonist which stimulates endogenous $S\text{-}100_B$ production, can be used to treat depression. A subject in need of treatment is administered an effective amount of $S\text{-}100_B$, a functional derivative

thereof, or a 5-HT_{1a} agonist. In adults, the treatment with S-100_B, or functional derivatives which do not cross the blood-brain barrier is by i.c.v. infusion. In neonates or immature subjects in whom the blood-brain barrier is not fully formed, the protein or derivative may be administered systemically. 5-HT_{1a} agonists, most of which readily cross the blood-brain barrier, are administered systemically.

Antidepressant agents have also been useful in the treatment of anxiety and obsessive-compulsive disorders (Gilman et al., supra), indicating the involvement of the serotonergic system in these states. In addition, 5HT is involved in synchronization of biological rhythms, and dysregulation may result in sleep disorders (Wauquier, A. et al., Ann. N.Y. Acad. Sci. 600:447-459 (1990)). According to the present invention, S-100_B, a functional derivative thereof, or a 5-HT_{1a} agonist may be used to treat anxiety and sleep disorders due to its action as an inducer of central serotonergic neuronal growth, as described above for treatment of depression.

Schizophrenia, a major psychiatric illness, is increasingly looked upon as a developmental disorder of the dopaminergic (DA) system wherein central DA activity is increased (Seene, P., Pharmacol. Rev. (1982)). The DA system appears to interact physiologically with serotonergic neurons in a way that stimulation of 5HT results in decrease in DA levels. Therefore, stimulation of growth of serotonergic neurons by administration of S-100_B or a functional derivative thereof according to the present invention may be useful in preventing or reversing the effects of enhanced dopaminergic activity, and thus the development of schizophrenia. In a preferred embodiment, S-100_B or a 5-HT_{1a} agonist which stimulates astroglial production and/or secretion of S-100_B is administered to a pregnant female carrying a fetus at risk for schizophrenia, either systemically or by intrauterine introduction.

Due to its action as a cortical growth factor, S-100_B promotes the growth and maintenance of cortical neurons,

most of which utilize glutamate as their neurotransmitter. According to the present invention, S-100$_B$, a functional derivative thereof, or a 5-HT$_{1a}$ receptor agonist may be used to induce repair of cortical neurons following cortical brain damage, such as that associated with traumatic head injury or stroke. Treatment of a subject in need of repair of cortical neurons is performed as described above for other diseases.

According to the methods of the present invention, the amounts and regimens for the administration of S-100$_B$ and functional derivatives thereof, 5-HT$_{1a}$ agonists and antagonists, and anti-S-100$_B$ agonistic and antagonistic antibodies can be determined readily by those with ordinary skill in the clinical art of treating the particular disease. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise about 0.001 to 100 mg/kg body wt. The preferred dosages comprise 0.1 to 10 mg/kg body wt.

As an alternative form of treatment to the administration of S-100$_B$ or 5-HT$_{1a}$ agonists, the present invention also contemplates the implantation or transplantation of an astroglial cell capable of producing S-100$_B$ to a subject having a deficit in such cells or having a genetic lesion rendering such cells non-functional, for example, non-responsive to 5-HT$_{1a}$ receptor stimulation. Such implanted cells may be derived from fetal or adult brain or may be a long term cell line, such as the C6 cell line (Labourdette, G. et al., supra), which is maintained in culture. Such cells can be implanted in specific target regions in the brain in order to stimulate serotonergic cell growth or cortical cell growth, as discussed above. (See, also: Azmitia, E.C. et al., Eds., Ann. N.Y. Acad. Sci., vol.495 (1987)).

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of

illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE I

$S\text{-}100_B$ IS A CNS SEROTONERGIC GROWTH FACTOR

METHODS

Brains from rat embryos (14 days; Taconia Farms, Germantown, NY) were dissected and the mesencephalic raphe region was removed and dissociated in Ca^{2+}/Mg^{2+} free Eagles' minimal essential medium (MEM; Sigma) by repeated trituration. The cells in complete medium (MEM supplemented with non-essential amino acids and 5% fetal calf serum) were plated in 96-well plates (Nunc Labware) coated with poly-L-lysine (25 μg/ml, Sigma) at initial plating densities (IPD) of 1 x 10^6 cells/cm^2.

After 3 days of incubation the cultures were washed and neuronal growth was assessed by the specific fluoxetine-sensitive high-affinity uptake of 50nM ^{3}H-serotonin for 20 min at 37°C in fresh MEM with 10^{-5}M pargyline (Azmitia, E.C. et al., Neuroscience 20:47-63 (1987)).

For morphometric analysis, cells were plated on 8-well glass culture chamber slides (Miles Scientific) for 30h, fixed with ice-cold 4% paraformaldehyde and reacted with a specific 5-HT antibody (1/4000 dilution; Incstar, Stillwater, MI) and an avidin-biotin secondary (Vector Labs). The 5-HT immunoreactive neurons were analyzed using a Bioquant computer imaging system. The slides were coded and all measures taken blind by a naive observer. On each well, 10 areas (0.5 mm^2) were randomly selected and the largest 5-HT-IR neuron in the field measured for somal area and neurite length (Azmitia, E.C., et al., 1987, supra).

The following protein preparations were dissolved in MEM, sterilized with 0.2 μm Uniflo filter units and added to the cultures at a 1/10 dilution: Human $S\text{-}100_{\beta}$, calmodulin (East-Acres Biologicals, Southbridge), NGF prepared from mouse submaxillary glands (Mobley, W.C. et al., Biochem.

15:5543 (1979)), EGF and insulin (Boehringer-Mannheim Biochemicals).

RESULTS

The daily chronic addition of S-100$_B$ produced a dose- dependent increase in 5-HT uptake capacity with maximal effect (171% of control) at 3.2 ng/ml and a significant increase (150% of control) seen at the lowest concentration tested of 1 pg/ml (Figure 1). Analysis of variance of the data of Figure 1 yielded the following values:

Comparison	df	F	p
S-100$_B$/Insulin	23	11.353	< 0.001
NGF/EGF	22	6.148	< 0.05

A post-hoc Tukey test showed + = $p < 0.05$ and ++ = $p < 0.01$.

Neither insulin, NGF nor EGF produced an increase in uptake capacity. A single application of S-100$_B$ at initial cell plating produced, after 3 days of incubation, a dose-related stimulation in uptake capacity (maximal stimulation of 85% at 5 ng/ml) while calmodulin was without effect (Figure 2). Analysis of variance for the data in Figure 2 showed df=23, F=24.3, and $p < 0.0001$. Students t-test control comparisons showed + = $p < 0.05$ and ++ = $P < 0.01$.

The uptake of [^{3}H]5-HT by the cultured serotonergic neurons is used as an index of total surface area of the neuron, However, it is possible that S-100$_B$ might interact directly with the 5-HT protein transporter and produce a change in the uptake of[^{3}H]5-HT independent of a increase in neurite length. Two experiments tested this possibility:

(1) S-100$_B$ (16 ng/ml) added for 15 min. to 3-day-old mesencephalic cultures immediately before [^{3}H]5-HT uptake produced no change (3140 $\pm$ 337 vs 3719 $\pm$ 177, S-100$_B$ and

control, respectively). This indicates that S-100$_B$ is not interacting directly with the 5- HT transporter.

(2) S-100$_B$ was added at initial plating for 30h to mesencephalic cultures and the 5-HT neurons were immunocytochemically stained. The neurite length of 5-HT-IR neurites was increased by 35% and 47% at two doses (16 and 3.2 ng/ml, respectively) of S-100$_B$ (Figure 3; ANOVA showed $F = 22.69$; $df = 20$ and $p < 0.0001$. Post-hoc Tukey test showed ++ = $p < 0.01$). No change in somal area was found. This increase in neurite length after 30h is consistent with the increase observed in [^{3}H]5-HT uptake by the cultured mesencephalic neurons after 3 days of incubation.

DISCUSSION

The results indicate that S-100$_B$ is an SGF. In contrast, EGF, insulin and calmodulin were not found to produce any stimulation, indicating that the SGF activity of S-100$_B$ was not a byproduct of its Ca^{2+} binding potential nor was it due to a general mitogenic potential.

S-100 in the brain is an astroglial specific protein (Isobe, T. et al., supra). Recent results have shown that the SGF properties of 5-HT$_{1a}$-stimulated, glial cell conditioned medium is blocked by treatment with an anti-S-100 antibody (see Example II, below).

It also possible that the soluble SGF detected several weeks after a 5,7-DHT lesion in adult hippocampus (Azmitia, E.C., et al., Soc. Neurosci. Abstr. 12 (1986)) and believed to be responsible for collateral sprouting of serotonergic nerves is also S-100$_B$.

EXAMPLE II

STIMULATION OF ASTROGLIAL 5-HT$_{1a}$ RECEPTORS RELEASES THE SEROTONERGIC GROWTH FACTOR, S-100$_B$

To test whether S-100 was the factor released by 5-HT$_{1a}$ receptor stimulation, astroglial cells were stimulated in primary culture with 100 nM ipsaperone (IPS), a 5-HT$_{1a}$ receptor agonist, and collected the conditioned media (GCM-IPS). We then added the GCM-IPS to primary cultures of

serotonin neurons, with and without the addition of an antibody to S-100, and assessed the effects on neuronal growth.

METHODS

Primary astroglial cultures were derived from newborn (1 to 3 day old) Sprague-Dawley rat pups as previously described (Whitaker-Azmitia, P.M. et al., Brain Res. 497:80-85 (1989)). After one week in culture, cultures were rinsed twice with Weymouth's medium containing 5 μg/ml insulin and 0.5 mg/ml albumin. The cells were left to incubate at 37°C. for twelve hours before replacing media with fresh serum-free media containing 100 nM ipsapirone, a selective 5-HT_{1A} receptor agonist. After 24 hr, the media (referred to as GCM-IPS) was collected and stored at -70°C until tested in neuronal cultures.

The growth-promoting properties of native bovine S-100 (10-1000 ng/ml and of GCM-IPS (diluted 1 to 500) were compared. The S-100 was obtained from East Acres Biologicals, Southbridge, MA (guaranteed >99% homogenous by SDS-PAGE).

S-100 or GCM-IPS, or each of these in the presence of a polyclonal antibody to S-100 (Accurate Chemical, Westbury, NY; final dilution 1/10,000) were added at the time of neuronal plating. The polyclonal antibody had been characterized in our laboratory and shown to be positive for immunocytochemical staining of astrocytes in culture and brain).

Mesencephalic neuronal cultures were prepared from Sprague-Dawley rat embryos at 13-14 days of gestation (obtained from Hilltop Breeding Laboratories) as previously described Azmitia, E.C. et al., Neuroscience 20:47-63 (1987). After three days in culture, neuronal growth was assessed by measurement of specific serotonin re-uptake capacity. This indicator has been shown to be a reliable measure of in vivo innervation density and of the maturational state of specific neurons in culture (Azmitia,

E.C., 1987, supra; Currie, D.N. et al., Brain Res. 199: 473-81 (1980); Dreyfus, C.F. et al., Brain Res. 128:124-139 (1977)). Briefly, cultures were incubated for 20 minutes with MEM containing 1% glucose and ^{3}H-serotonin (26 Ci/mmole, New England Nuclear; final concentration 50 nM) with or without 50 nM fluoxetine. After removing the radiolabel, the cultures were allowed to dry and 200 microliters of absolute ethanol was added for one hour. Then, 150 µl of the sample was placed into 7 ml of Liquiscint for counting in a Beckman Liquid Scintillation Counter (40% efficiency). Test cultures were pre-incubated with S-100 or GCM-IPS for one hour before uptake was measured.

To visualize astroglial cultures after exposure to serum-free media with or without 100 nM ipsapirone, cultures were rinsed twice with Tris-buffered saline (TBS) at 4°C before incubation with a polyclonal antibody to a specific astroglial marker, glial fibrillary acidic protein (GFAP) (Accurate Chemicals; final dilution 1/800 in TBS with 0.2% Triton and 0.1% normal swine serum) for 2 hrs at 37°C. After rinsing with TBS, the cultures were stained using the avidin/biotin method prepared as Vectastain (Vector Labs) with final visualization using diaminobenzidine.

RESULTS

Both S-100 (500 ng/ml) and GCM-IPS (diluted 1/500) produced an increase in the ^{3}H-serotonin uptake capacity of the cultures after 3 days of exposure, but not when applied acutely. This stimulation was blocked by incubation, at the time of plating, with a polyclonal antibody to S-100 at a 1/10,000 dilution (Figure 4; df=5; f=1497, $p < .0001$; for individual values, $p < .001$). Application of either the antibody alone or 0.5 nM ipsaperone(the maximum final concentration in the neuronal cultures after addition of GCM-IPS) was without significant effect. The majority of the activity is thought to reside in the ß subunit of S-100 since $S\text{-}100_B$ (ß-ß dimer) is more active than $S\text{-}100_A$ (ß-α dimer).

The morphological alterations were characterized by an increase in process-bearing cells and an increased colonization of the cells. These changes were consistently observed in all eight primary cultures (ie. from eight different litters of animals) used to derive GCM.

DISCUSSION

It had been previously demonstrated that serotonergic neurons regulate their own growth through activation of a 5-HT_1 receptor (Whitaker-Azmitia, P.M. et al., Neurosci. Lett. 67:307-312 (1986)). The present inventors found that astroglial cells contain high levels of 5-HT_1 receptors in the immature state (Whitaker-Azmitia, P.M. et al., J. Neurochem. 46:1186-91 (1986)) and that activation of a subtype of these receptors, the 5-HT_{1a} receptor, leads to secretion into the medium of a factor which can stimulate serotonergic maturation in dissociated tissue culture preparation (Whitaker-Azmitia, P.M. et al., Brain Res. 497:80-85 (1989)).

Based on the above results, 5-HT_{1a} receptors on brain astroglial cells appear to be involved in the release of S-100. Therefore, S-100 provides at least one means by which serotonin can autoregulate development of serotonergic nerves.

During fetal brain development, S-100, as detected by antibodies, shows an intense yet transient rise in the midline raphe region, where the serotonin cells are developing (Van Hartesveldt, C.J. et al., J. Comp. Neurol. 253:175-184 (1986)). Since in the process of producing and/or releasing S-100, the astroglial cells attain a mature morphology, the present results suggest a functional interaction between astrocytes and neurons during development, whereby both cell types mature through the action of the astroglial 5-HT_{1a} receptor.

The observed morphological change has also been seen after activation of other receptors linked to generation of cyclic AMP, such as the ß-adrenergic receptor,

the stimulation of which is linked to the release of nerve growth factor (NGF) (Schwartz, J.P. et al., Naunyn Schmeideberg's Arch. Pharmacol. 300:123-129 (1977)). The parallels of these two systems is noteworthy: both involve receptor stimulation coupled to cAMP production which results in astroglial morphological changes and finally release of a growth factor.

In summary, of the several growth factors tested, only chronic S-100$_B$ showed enhancement (maximal at 3.2 ng/ml is 171%) after 3 days of incubation of the [^{3}H]5-HT uptake capacity by serotonergic neurons. A single application at initial plating of S-100$_B$ (maximal at 5 ng/ml is 185%), but not calmodulin, increased the development of the [^{3}H]5-HT uptake capacity by the cultured serotonergic neurons. Morphometric analysis of cultured 5-HT immunoreactive (IR) neurons showed an increase (135 and 147%) in neurite length 30 h after S-100$_B$ application of 16 and 3.2 ng/ml (respectively). The results indicate that S-100$_B$ functions as a serotonergic growth factor in the mammalian brain.

Stimulation of astroglial 5-HT$_{1a}$ receptor causes astroglial cells to acquire a more mature morphology and to release a factor (or factors) which promotes growth of serotonergic neurons. By using an antibody- blocking approach, it has been conclusively demonstrated that one of the growth- factors released is the astroglial-specific protein S-100. This may be a particularly important observation, in view of studies implicating S-100 in both Down's Syndrome and Alzheimer's Disease, as discussed above.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for stimulating the production or release of S-100$_B$ in a subject, comprising administering to said subject an effective amount of an agonist acting at the 5-HT$_{1a}$ receptor.

2. The method of claim 1 wherein said agonist is selected from the group consisting of 5-hydroxytryptamine, 5-methoxytryptamine, buspirone, 8-hydroxydipropylaminotetralin, ipsaspirone, gepirone, SM23997, lysergic acid diethylamide, and an agonistic antibody.

3. A method for stimulating growth of central cortical or serotonergic neurons in a subject comprising administering to said subject an effective amount of S-100$_B$, a functional derivative thereof, or an agonist acting at the 5-HT$_{1a}$ receptor.

4. The method of claim 3 wherein said agonist is selected from the group consisting of 5-hydroxytryptamine, 5-methoxytryptamine, buspirone, 8-hydroxydipropylaminotetralin, ipsaspirone, gepirone, SM23997, lysergic acid diethylamide, and an agonistic antibody.

5. A method for stimulating growth of central cortical or serotonergic neurons comprising contacting said neurons with an effective amount of S-100$_B$ or a functional derivative thereof.

6. The method of claim 5 wherein said contacting is *in vitro*.

7. The method of claim 5 wherein said contacting is in vivo.

8. A method for inhibiting the growth of central serotonergic neurons comprising contacting said neurons with an effective amount of an inhibitor of S-100_B production or action.

9. The method of claim 8 wherein said inhibitor is an antibody specific for S-100_B or an antagonistic antibody specific for the 5-HT_{1a} receptor.

10. The method of claim 9 wherein said antibody is a polyclonal antibody.

11. The method of claim 9 wherein said antibody is a monoclonal antibody.

12. The method of claim 8 wherein said inhibitor is a 5-HT_{1a} receptor antagonist.

13. The method of claim 12 wherein said antagonist is selected from the group consisting of spiperone and spiroxatine.

14. A method for treating a disease associated with decreased central serotonergic innervation or activity in a subject comprising administering to said subject an effective amount of S-100_B, a functional derivative thereof or a 5-HT_{1a} agonist.

15. The method of claim 14 wherein said agonist is selected from the group consisting of 5-hydroxytryptamine, 5-methoxytryptamine, buspirone, 8-hydroxydipropylamineotetralin, ipsaspirone, gepirone, SM23997, lysergic acid diethylamide and an agonistic antibody.

16. The method of claim 14 wherein said disease is selected from a group consisting of autism, depression, anxiety, biological rhythm-based sleep disorder, and cortical brain damage.

17. A method for treating a disease associated with increased central serotonergic innervation or activity in a subject comprising administering to said subject an effective amount of an inhibitor of $S\text{-}100_B$ production or action.

18. The method of claim 17 wherein said inhibitor is an antibody specific for $S\text{-}100_B$.

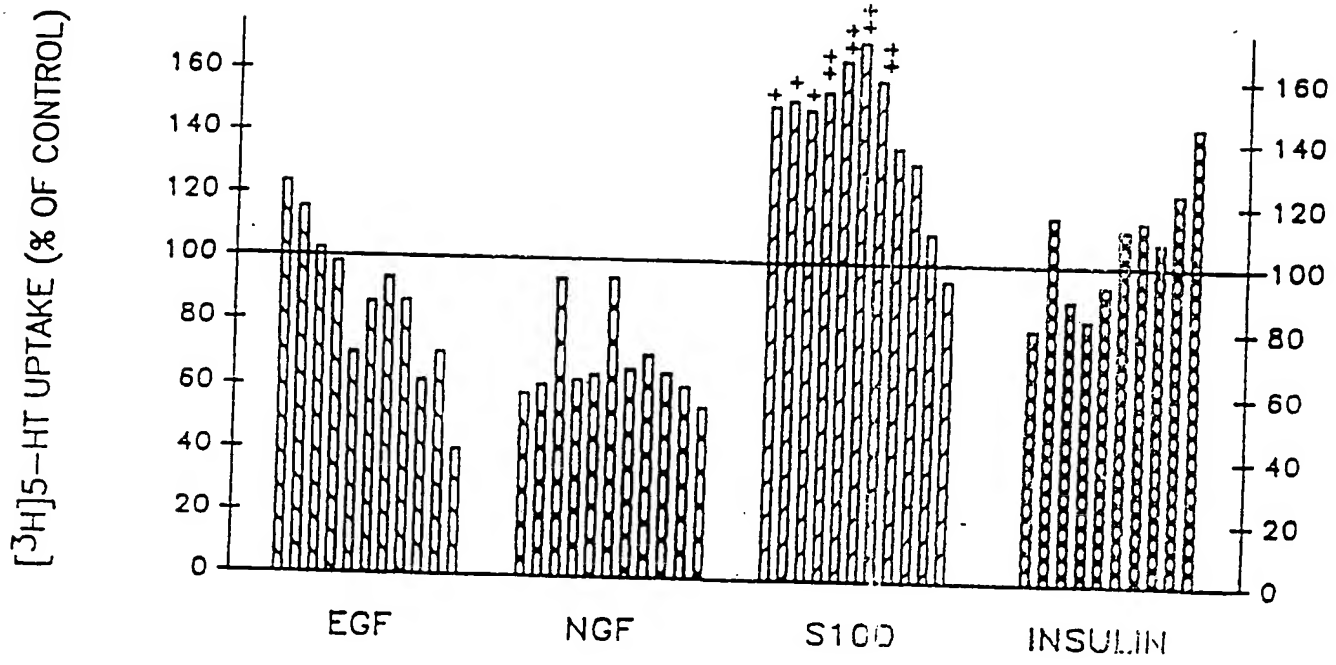
19. The method of claim 17 wherein said inhibitor is a $5\text{-}HT_{1a}$ receptor antagonist.

20. A method for stimulating cortical neuronal growth in a subject having Alzheimer's disease comprising the steps of:

(a) up-regulating the expression of $5HT_{1a}$ receptors on astroglial cells in the brain of said subject; and

(b) stimulating the release of $S\text{-}100_B$ in said subject according to the method of claim 1,

thereby stimulating said cortical neuronal growth.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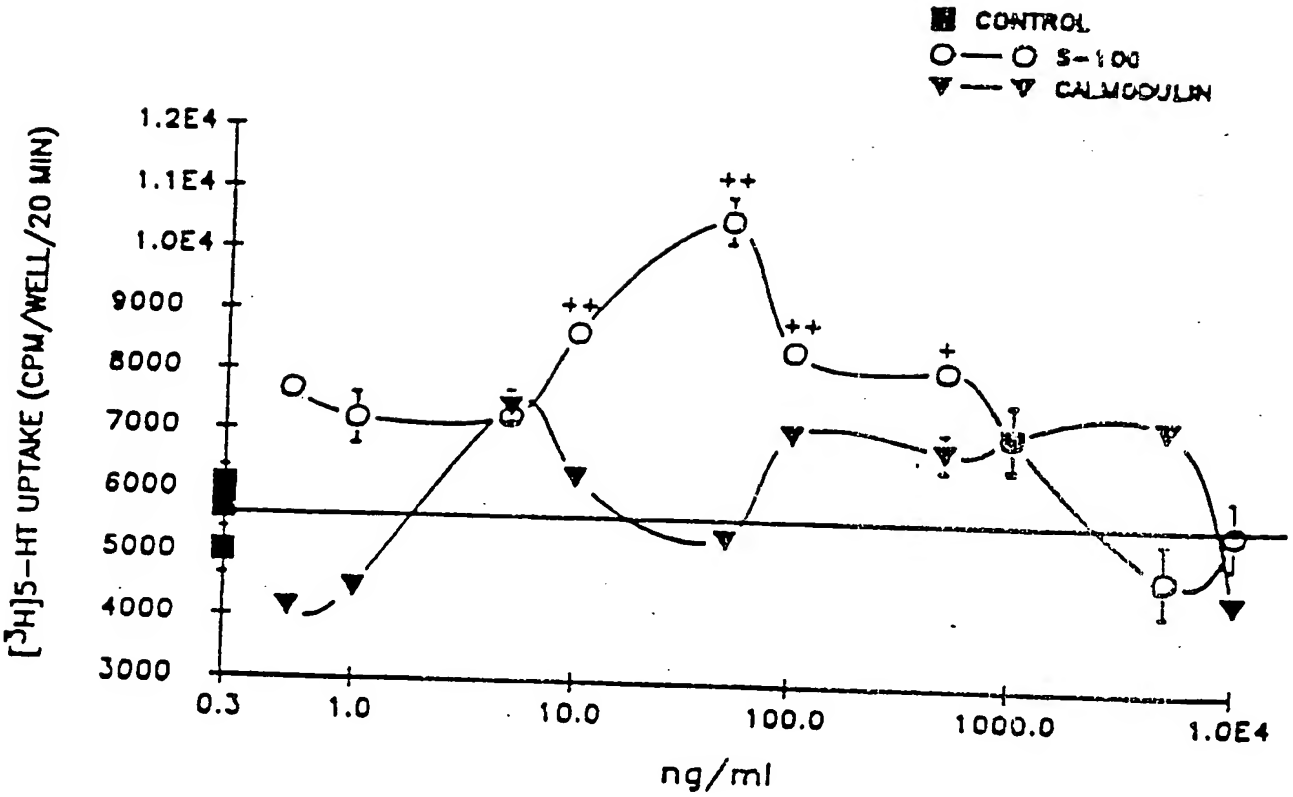
140
120
100
80
60
40
20
0
EGF
NGF
S100
INSULIN


CONTROL
S-100
CALMODULIN
[3H]5-HT UPTAKE (CPM/WELL/20 MIN)
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1.1E4
1.0E4
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5000
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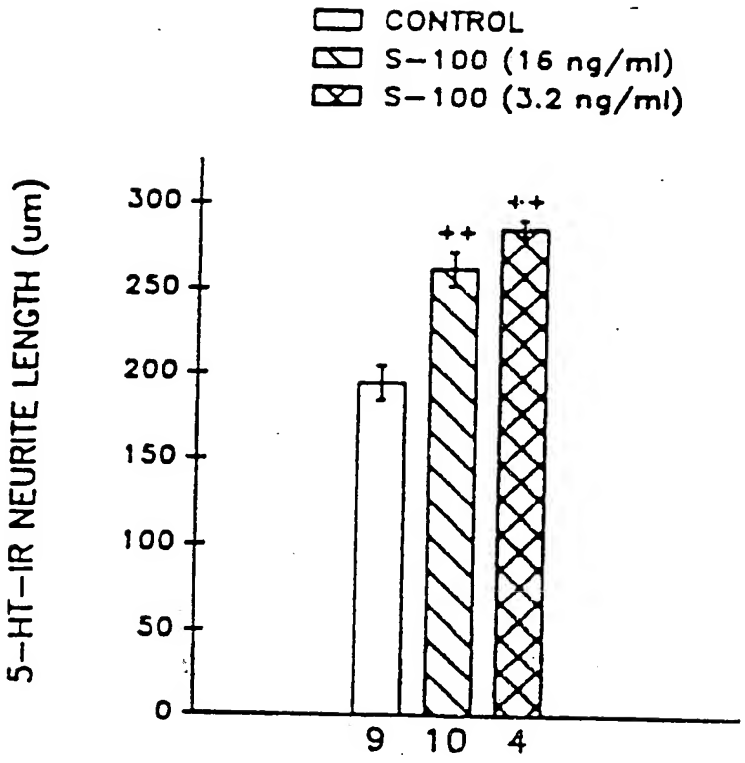
3000
0.3
1.0
10.0
100.0
1000.0
1.0E4
ng/ml


CONTROL
S-100 (16 ng/ml)
S-100 (3.2 ng/ml)
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200
150
100
50
0
++
++
9
10
4

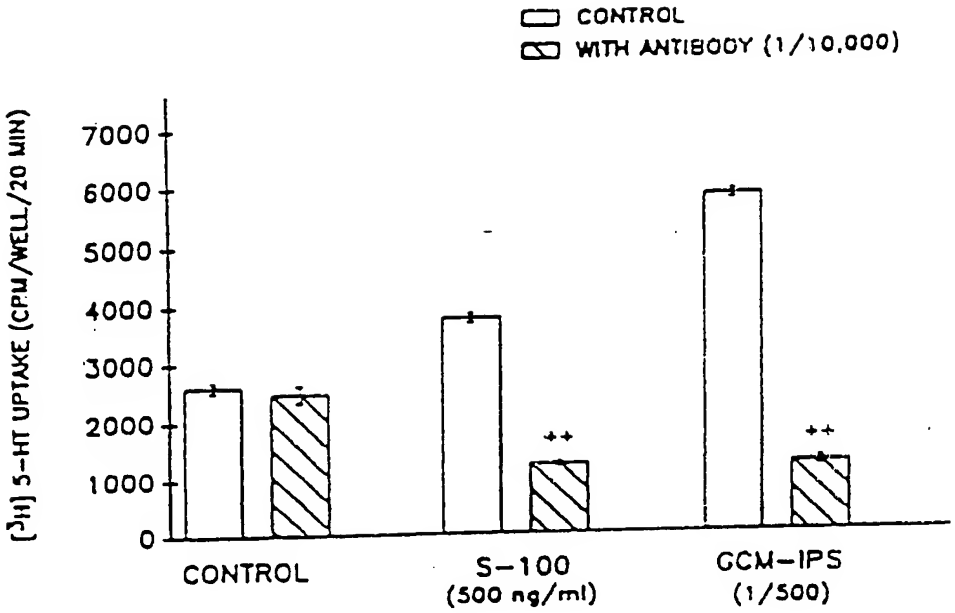
CONTROL
WITH ANTIBODY (1/10,000)
[3H] 5-HT UPTAKE (CPM/WELL/20 MIN)
7000
6000
5000
4000
3000
2000
1000
0
++
++
CONTROL
S-100
(500 ng/ml)
GCM-IPS
(1/500)

Fig. 4

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/09383

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61 K 37/00, 39/00, 35/14, 31/54, 31/495
US CL : 514/12; 424/85.8; 530/387; 514/225, 255

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
	514/12; 424/85.8, 530/387; 514/225, 255

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

Automated Patent System, Chemical Abracts, Biosis, and Medline Commercial Databases

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X/Y	The Journal of Cell Biology, Volume 109, issued 14 December 1989, F. Winningham-Major et al., "Neurite Extension and Neuronal Survival Activities of Recombinant S100.beta Proteins that Differ in the Content and Position of Cysteine Residues", pages 3063-3071. See especially p. 3064, and paragraph and p. 3065 "Effects of VUSB-1 on Neurite Extension" Section.	3,5,6/ 3,5,7
X/Y	Brain Research, Volume 516, issued 1990, E.C. Azmitia et al., "S-100.beta but not NGF, EGF, insulin or calmodulin is a CNS Serotonergic growth factor", pages 354-356. See entire document.	3,5,6/ 3,5,7-11
Y	Proceedings National Academy of Sciences, USA, Volume 81, issued October 1984, L.J. Van Eldik et al., "Production and characterization of monoclonal antibodies with specificity for the S100.beta polypeptides of brain S100 fractions", pages 6034-6038. See especially p. 6034, second column, first full paragraph.	8-11

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
20 MARCH 1992	02APR 1992
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	Susan Perkins

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET		
A	Journal of Neurochemistry, Volume 46, No. 4, issued 1986, P.M. Whitaker-Azmitia et al., "[3H]5-Hydroxy-tryptamine Binding to Brain Astroglial Cells: Differences Between Intact and Homogenized Preparations and Mature and Immature Cultures" pages 1186-1189. See Abstracts.	1-20
X	US, A, 4,423,049 (Temple, Jr.) 27 December 1983. See Abstract and col. 7, lines 17-30.	14-16
X	US, A, 4,612,312 (Hibert et al) 16 September 1986, See Abstract and col. 1, lines 35-44.	14-16
X	US, A, 4,507,303 (Ishizumi et al) 26 March 1985. See Abstract and col. 1, lines 1-13.	14-16

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
A	US, A, 4,654,313 (Hartman) 31 March 1987. See Abstract.	1-20
X	"The Pharmacological Basis of Therapeutics Goodman and Gilman's" published 1990, page 428-429. See especially p. 428, column 1 and Table 18-5.	14-16